Deoxyglucose-6-Phosphate Stability In Vivo and the Deoxyglucose Method: Response to Comments of Hawkins and Miller

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We recently reported that claims of the invalidity of the 2-deoxy-D-glucose (DG) method due to glucose-6-phosphatase (EC 3.1.3.9; G-6-Pase) activity in brain were without foundation (Nelson et al., 1986*a*). Hawkins and Miller refute none of the evidence in our report but adhere to their previous opinion, which they support with a wide array of arguments. Most of their arguments are based on misinterpretations of experimental observations, disregard of explicit facts, or speculations; none provides convincing evidence that the DG method, when used as prescribed, is seriously flawed by the effects of G-6-Pase activity. Space constraints allow us to respond only to those that appear to be most relevant and substantive.

GLUCOSE-6-PASE ACTIVITY IN BRAIN IN VITRO

The presence of small amounts of G-6-Pase in brain has been well established by biochemical and histochemical techniques (see citations by Hawkins and Miller). Hawkins and Miller's Table 1 lists reported V_{max} values based on in vitro enzyme assays usually carried out under optimal conditions [e.g., saturating concentrations of substrate; pH 6.5–6.6; presence of enzyme-activating detergent that breaks down membrane barriers and compartmentation; and absence of endogenous inhibitors such as glucose, citrate, bicarbonate, phosphate, ATP, etc. (Nordlie, 1974)]. None of these optimal conditions exist in vivo. Nordlie (1974) found that liver G-6-Pase activity in vitro is inhibited about 70% by a combination of some of the endogenous inhibitors at concentrations simulating "physiological conditions." Enzyme activities in vivo rarely equal maximal activities assayed under optimal conditions in vitro. For example, brain hexokinase in vivo normally operates at 2–3% of its maximal activity (Lowry and Passonneau, 1964); G-6-Pase activity probably behaves similarly. It should be emphasized that it is misleading to extrapolate from V_{max} values in vitro to predicted effects of G-6-Pase activity on 2-deoxy-D-glucose-6-phosphate (DG-6-P) in brain in vivo (Hawkins and Miller's Fig. 1).

G-6-PASE ACTIVITY IN BRAIN IN VIVO

Brain labeled in vivo with [^{14}C]DG is not permanently labeled. The label is eventually lost, and [^{14}C]DG-6-P is lost from brain in vivo only after hydrolysis. The possible effects of this loss were carefully considered during the development of the DG method. We first attempted to estimate local rates of [^{14}C]DG-6-P loss from brain in vivo from the clearances of ^{14}C from local brain regions after arterial

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Abbreviations used: DG, 2-deoxy-D-glucose; DG-6-P, 2-deoxy-D-glucose-6-phosphate; G-6-P, glucose-6-phosphate; G-6-Pase, glucose-6-phosphatase; ^{18}FDG, 2-[^{18}F]fluoro-2-deoxy-D-glucose; LCMRglu, local cerebral glucose utilization; PET, positron emission tomography.

plasma concentrations of [^{14}C]DG had fallen to levels sufficiently low to minimize reincorporation from plasma, i.e., 17–24 h, after a pulse of [^{14}C]DG. We assumed that the loss of label from brain was rate limited by a constant first-order rate of hydrolysis of [^{14}C]DG-6-P. The results indicated half-lives for [^{14}C]DG-6-P of 8–10 h (Sokoloff et al., 1977). We later learned that some [^{14}C]DG was also incorporated into glycogen (Nelson et al., 1984); the half-lives at 17–24 h probably reflected the slower clearances of label from glycogen and other compounds and underestimated the rate of [^{14}C]DG-6-P loss. The design of the procedure of the DG method was not based, however, on these estimates of [^{14}C]DG-6-P loss. It was obvious that G-6-Pase activity would cause loss of product and that the fractional loss of total product formed would increase with time after the pulse of [^{14}C]DG. If significant, this loss would lead to erroneously low estimates of local cerebral glucose utilization (LCMRglu) that would become even lower with time. We therefore tested the DG method with experimental periods of various durations and selected 45 min as the recommended maximal duration of the experimental period because the anticipated effects of G-6-Pase activity could not be detected during this period (Sokoloff et al., 1977).

TIME COURSE OF EFFECTS OF G-6-PASE ACTIVITY IN VIVO

Studies with the [^{14}C]DG method applied over various experimental periods between 20 and 120 min showed that the magnitude of the effect of G-6-Pase activity in vivo varied with time; it is insignificant within the first 45 min after the pulse of [^{14}C]DG, becomes apparent by 60 min, and then increases with time (Fig. 1; Table 1) (Sokoloff et al.,

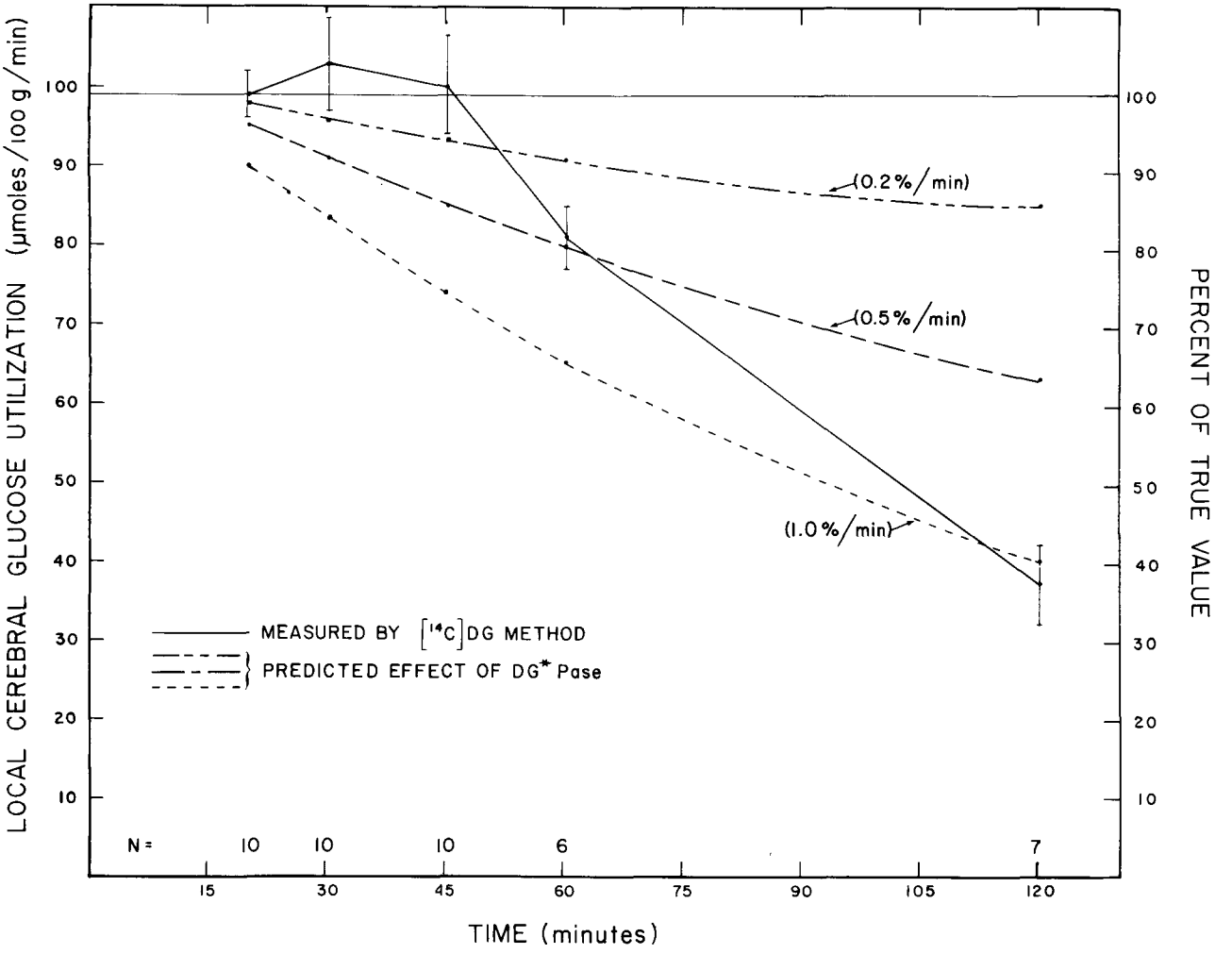

FIG. 1. Effect of duration of experimental period on values for local cerebral glucose utilization in gray matter (arithmetic mean of values in gray structures) calculated with the DG operational equation (Fig. 2B), which assumes no loss of DG-6-P due to G-6-Pase (solid line). The points and vertical bars at 20, 30, 45, 60, and 120 min represent means ± SEM obtained in the number of animals indicated by N. The broken lines represent the values for glucose utilization that would have been obtained with the operational equation had there been a continuous loss of DG-6-P due to G-6-Pase activity, or any other cause, at rates of 0.2%, 0.5%, or 1.0% per minute. From Sokoloff (1982).

TABLE 1. *Comparison of values of LCMRglu determined at 30 and 45 min following pulse of [^{14}C]DG in the normal conscious rat*

Structure	30 min (10)[a] (μmol/100 g/min)	45 min (10)[a] (μmol/100 g/min)
	Gray matter	
Visual cortex	113 ± 4	107 ± 6
Auditory cortex	163 ± 4	162 ± 5
Parietal cortex	110 ± 3	112 ± 5
Sensory-motor cortex	124 ± 3	120 ± 5
Olfactory cortex	104 ± 2	98 ± 5
Frontal cortex	112 ± 4	116 ± 5
Thalamus: lateral nucleus	110 ± 2	116 ± 5
Thalamus: ventral nucleus	98 ± 2	109 ± 5
Medial geniculate body	130 ± 4	131 ± 5
Lateral geniculate body	94 ± 2	96 ± 5
Hypothalamus	61 ± 2	54 ± 2[b]
Mammillary body	123 ± 4	121 ± 5
Hippocampus: Ammon's horn	79 ± 1	79 ± 3
Hippocampus: dentate gyrus	70 ± 2	67 ± 3
Amygdala	54 ± 2	52 ± 2
Septal nucleus	62 ± 1	64 ± 3
Caudate-putamen	111 ± 2	110 ± 4
Nucleus accumbens	83 ± 3	82 ± 3
Globus-pallidus	58 ± 3	58 ± 2
Substantia nigra	61 ± 2	58 ± 3
Vestibular nucleus	134 ± 3	128 ± 5
Cochlear nucleus	128 ± 5	113 ± 7
Superior olivary nucleus	147 ± 6	133 ± 7
Lateral lemniscus	114 ± 5	104 ± 5
Inferior colliculus	203 ± 6	197 ± 10
Superior colliculus	100 ± 3	95 ± 5
Pontine gray matter	69 ± 2	62 ± 3
Cerebellar cortex	63 ± 2	57 ± 2
Cerebellar nucleus	106 ± 3	100 ± 4
	White matter	
Corpus callosum	39 ± 2	40 ± 2
Genu of corpus callosum	35 ± 3	34 ± 1
Internal capsule	33 ± 2	33 ± 2
Cerebellar white matter	37 ± 1	37 ± 2

From Sokoloff et al. (1977).

[a] The values are the means ± standard errors from measurements made in the number of animals indicated in parentheses.

[b] Statistically significant difference ($p < 0.05$).

1977; Sokoloff, 1982). The operational equation of the method (Fig. 2B) assumes no loss of DG-6-P. If there were significant loss, calculated rates of glucose utilization would be too low and would decrease progressively with increasing experimental periods because the product loss is cumulative while the rate of synthesis of [^{14}C]DG-6-P diminishes as the [^{14}C]DG concentration declines with time after a pulse. Values obtained with the DG method at 30–45 min are not low; average values of about 68 and 36 μmol/(100 g · min) for rat and monkey brain, respectively (Sokoloff, 1985) agree with values for cerebral energy metabolism measured with the Kety–Schmidt and other methods (Siesjö, 1978). Also, local rates in discrete cerebral structures do not decline significantly during the first 45 min; 33 structures of rat brain were examined in the study summarized in Table 1, and only one showed a significant difference ($p < 0.05$) between 30 and 45 min (Sokoloff et al., 1977).

The lack of a significant time dependence in LCMRglu determined with the DG method between 20 and 45 min has been confirmed in three other series (Sokoloff, 1982; Mori et al., 1986). Figure 1 (reproduced from Sokoloff, 1982) shows that there are no significant differences in LCMRglu values at 20, 30, and 45 min for rats studied with the DG method; at 60 min there is a small decline, which increases between 60 and 120 min. If there were a constant fractional rate of loss of product throughout the experiment (i.e., a k_4^*, a first order rate constant for product loss due to dephosphorylation), the DG operational equation (which assumes no loss) would yield erroneously low values for glucose utilization that would decline with time. Figure 1 also contains the theoretical time courses that the operational equation would have yielded for the values of LCMRglu in the same animals if there had actually been continuous losses of product at rates of 0.2%, 0.5%, or 1.0% per minute during the entire experiment (broken lines). The broken lines show that if there were product loss, the operational equation would yield low values for LCMRglu; the larger the rate of loss, the lower the values at all times and the more rapid their decline with time. The solid line represents the actual experimental values for LCMRglu that were obtained with the operational equation; it intersects all the broken lines but at different times, the greater the assumed rate of loss, the later the intersection. The points on the solid line at 20, 30, and 45 min are all above the broken lines, even the one for the lowest assumed rate of loss, 0.2%/min, and do not change significantly with time; these observations indicate that the rate of loss over this period is no greater and probably less than 0.2%/min and is neither significant nor detectable. The solid line crosses the broken line representing 0.2% loss/min between 45 and 60 min, falls to about the level of the broken line representing 0.5% loss/min at 60 min, and finally falls to the level of the broken line for 1.0%/min at about 120 min. The rate constant for the loss of labeled product (i.e., k_4^*) is thus time dependent. Because its effects are undetectable during the first 45 min after the pulse, k_4^* is not significantly different from zero during this period although because of the variances in the data the possibility of some loss, somewhere between 0% and 0.2%, cannot be excluded. After 45 min the loss increases progressively until at 2 h it equals the loss that would have resulted from a constant rate of loss of 1%/min. The fractional rate of loss during each segment of time at first increases after 45 min and then decreases as the

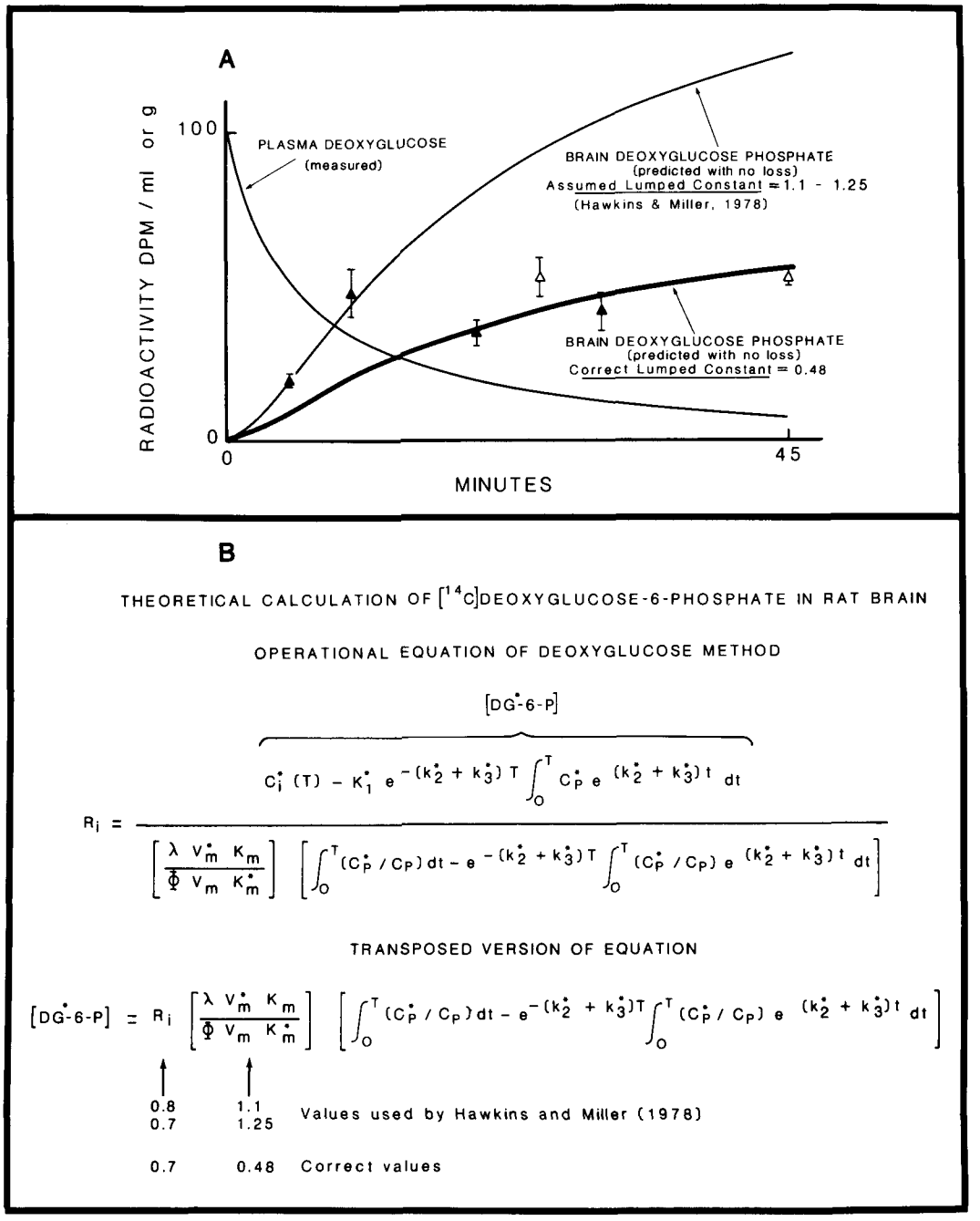

FIG. 2. A: Comparison of time courses of [^{14}C]DG-6-P accumulation in rat brain measured chemically (triangles) and computed (continuous lines) by a transposed version of the DG operational equation, which assumes no loss of product due to G-6-Pase activity. Mean ± SEM for 4–12 rats (▲); mean ± range of 2 rats (△). Modified from Fig. 1 of Hawkins and Miller (1978) by removing the circles representing free [^{14}C]DG concentrations in brain and by adding the heavy solid line representing predicted [^{14}C]DG-6-P calculated with the 0.48 lumped constant. From Sokoloff (1979, 1980, 1982). **B:** The DG operational equation and its transposed version used to calculate the theoretical curve in Fig. 2A.

proportion of label sequestered in slowly turning over components, such as glycogen, increases. This relatively long lag before the effects of G-6-Pase activity become apparent is not surprising; it reflects the summation of a series of lags. There must be in sequence: (1) delivery of DG to brain tissue and buildup of the precursor pool; (2) DG phosphorylation and accumulation of DG-6-P in the cytosol; (3) diffusion of DG-6-P across the endoplasmic reticular membrane; (4) hydrolysis of the DG-6-P in the cisterns of the endoplasmic reticulum; and (5) loss of the released DG from the tissue. Some of the released DG

is almost certainly reincorporated. The passage of DG-6-P across the endoplasmic reticular membrane may well be the slowest step. Fishman and Karnovsky (1986) recently showed that the translocase, which transports hexose-6-phosphates from cytosol (where they are formed) into the cisterns of the endoplasmic reticulum (where the G-6-P hydrolase resides), is not present in brain as it is in liver and other organs where G-6-Pase activity is high. The rate of DG-6-P hydrolysis in brain may be limited, therefore, not by the amount of G-6-Pase but by the diffusion of DG-6-P through the endoplasmic reticular membrane and the accessibility of the substrate to the enzyme. In organs, such as liver, with significant G-6-Pase activity the genetic absence of the translocase is sufficiently rate limiting to produce a glycogen storage disease (type 1b) similar to that resulting from genetic absence of the G-6-P hydrolase (Lange et al., 1980). It is noteworthy that the genetic absence of either the hydrolase or the translocase has no direct metabolic consequences in brain.

We do not claim that the lag is absolute and that there is zero loss of DG-6-P from brain for 45 min after which the loss is suddenly turned on. The loss is continuous; it begins slowly and gradually increases. There is certainly some loss of DG-6-P during the first 45 min, but it is so small that its effect is negligible (i.e., within the variances due to random biological and methodological variations) and hardly enough to invalidate the DG method. After 45 min the effects of DG-6-P loss do become significant, and correction for the loss of DG-6-P can and should be made. The determination and use of such a correction was first reported by Phelps et al. (1979), which Hawkins and Miller use as evidence for the invalidity of the DG method because of loss of product. It should be noted that the k_4^* (0.68%/min) used for the correction was determined over several hours and used in studies extending over hours, rather than the prescribed 45 min, because of the slowness of the positron emission tomography (PET) scanners used with the 2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}FDG) adaptation of the method for human use. In most of the citations in Hawkins and Miller's Table 2 the rates of product loss were determined from measurements made at periods >45 min. In the study by Deuel et al. (1985), done with nuclear magnetic resonance and gas chromotography in the rat, the losses of DG-6-P were seen only at late times with a time course similar to the one in our present Fig. 1 (Nelson et al., 1986*b*). Hawkins and Miller point out that the rate of decline during that period is linear; this only confirms that the k_4^* must increase with time (i.e., a constant rate of loss from a diminishing pool of [^{14}C]DG-6-P).

DIRECT BIOCHEMICAL EVIDENCE IN VIVO

Direct evidence that G-6-Pase activity has no detectable effect on DG-6-P accumulation in brain during the prescribed 45 min of the DG procedure was, in fact, first provided by Hawkins and Miller (1978). They applied the DG procedure to rats, which were killed by freeze-blowing the brain (Veech et al., 1972) at various times between 5 and 45 min, and chemically measured the brain [^{14}C]DG-6-P concentrations. Using the transposed version of the operational equation of the DG method (Fig. 2B), they calculated a time course of [^{14}C]DG-6-P accumulation in brain that should have occurred if there were no loss of [^{14}C]DG-6-P. This calculation required values for the lumped constant (i.e., a proportionality factor for relative rates of uptake and phosphorylation of DG and glucose by brain from plasma) and for the average rate of glucose utilization in whole rat brain. They assumed a reasonable value for rat cerebral glucose utilization, 0.7–0.8 μmol/(g · min), but used 1.1–1.25 for the lumped constant instead of the reported 0.48, which was measured directly with a model-independent steady-state method (Sokoloff et al., 1977). Their theoretical values for [^{14}C]DG-6-P accumulation in brain were therefore more than twice their measured values (Fig. 2A), a difference they attributed to the loss of [^{14}C]DG-6-P due to G-6-Pase activity. Their choice of a lumped constant of 1.1–1.25 resulted from a series of compounded errors described in our recent report (Nelson et al., 1986*a*). When the theoretical curve was redetermined with the measured lumped constant of 0.48, the predicted and measured values agreed remarkably well through all but the very earliest part of the time course (Fig. 2A); in contrast with their conclusion, this recalculation indicates no detectable loss of DG-6-P during the 45-min experimental period of the DG method. The discrepancy at early times is expected because of heterogeneity effects in freeze-blown brain tissue as discussed below.

VALUE OF THE LUMPED CONSTANT

Hawkins and Miller argue that the lumped constant of 0.48, determined from steady-state extractions of DG and glucose by brain from blood, is too low because, according to them, the net extraction of [^{14}C]DG decreases with time as a result of G-6-Pase activity, thus reducing the apparent value of the constant. To support this claim they describe Fig. 4 in the original report of the DG method (Sokoloff et al., 1977) as follows (Hawkins and Miller's footnote 2): "Close examination of Figure 4A from Sokoloff et al. (1977) shows decreasing values for arteriovenous differences across rat brain as time passed, and Fig. 4B in the same article shows lower and lower values for the 'lumped constant'." That statement is inaccurate. Their "close examination" failed to discern the following explicit statement in that same paper (Sokoloff et al., 1977) that explained the theory and procedure for determining the lumped constant: "Equations (38) and (40) prescribe the procedure to determine the lumped constant. They state that if

[^{14}C]DG is so administered to the animal that C_p^* is maintained constant long enough for the exponential factor

$$e^{-(k_2^*+k_3^*)T}$$

to approach zero, then the ratio of the fractional extractions of [^{14}C]DG and glucose by the brain multiplied by the ratio of the specific activities (i.e., ratio of [^{14}C]DG to glucose concentrations) in arterial blood and plasma declines exponentially with a rate constant equal to $(k_2^* + k_3^*)$ until it reaches an asymptotic value equal to the lumped constant." That is exactly what Fig. 4 in the article shows. Hawkins and Miller confused the lumped constant with the function that declines exponentially with time and only at its steady-state asymptotic value becomes equal to the lumped constant. The lumped constant in Fig. 4B of that article is represented by the last four points between 20 and 40 min; these points show only random variation and no systematic decline with time. Other examples of an unvarying lumped constant during the steady state were provided in Fig. 6 of Nelson et al. (1986*a*) and Fig. 3 of Sokoloff (1982). The lumped constant for each animal was calculated as the mean of all the individual values during the steady state; several values distributed over time were obtained in each experiment to ensure the existence of a steady state, which was usually achieved within 20 min and persisted beyond 40 min. The statement in Hawkins and Miller's footnote 2, "Since the data shown were from single selected animals, statistical analyses are, of course, impossible," is incorrect. Several values for the lumped constant during the steady-state period were obtained for each animal, and statistical analysis was performed. Hawkins and Miller failed to consider the following statement in the legend to Fig. 6 of Nelson et al. (1986*a*): "When we compute the correlation coefficient between the calculated lumped constant at individual time points obtained in all of the animals with time over the steady state period of 20–45 min, no significant correlation with time is found ($r = -0.219$, $p = 0.676$)." This analysis was applied to data for 15 conscious rats. There is no evidence that the lumped constant, determined by the steady-state procedure, is measurably diminished by G-6-Pase activity during the 20–45 min period of measurement.

Hawkins and Miller cite a report by Hargreaves et al. (1986), who indirectly computed a lumped constant of 0.65 (no SD or SEM provided) from experimental estimations of components comprising the lumped constant, and speculated that the difference between their value and the directly measured steady-state value of 0.48 might be explained if there were a continuous 1%/min loss of DG-6-P during the 45 min steady-state procedure. Hargreaves et al. (1986) also incorrectly assumed that the value of 0.48 for the lumped constant was determined only at 45 min; as stated above, the lumped constant was determined for each animal as the mean of several values obtained throughout the steady-state period, which always began between 10 and 30 min and extended to 40–45 min. Because of variances in the lumped constant determined by both methods, it is unlikely that the two values, 0.48 (SD = ±0.11) and 0.65, are significantly different. Two of the same authors (Cunningham and Cremer, 1981) previously measured the lumped constant with a similar method and found values in various brain regions between 0.45 and 0.70; they concluded that the values were "in *good agreement* (our emphasis) with the values obtained for the whole brain by Sokoloff et al. (1977)." Also Gjedde (1982), Gjedde and Diemer (1983), Diemer and Gjedde (1983), and Crane et al. (1981) have found values for the lumped constant between 0.4 and 0.6 in normoglycemic rats.

EFFECTS OF HETEROGENEITY

In Fig. 3 of their response, Hawkins and Miller reproduce Figs. 1A and 2 of Nelson et al. (1986*a*) but add calculated values for apparent CMRglu relative to the calculated value at 45 min. These calculated relative values for CMRglu are higher at early times and decrease with time. This time-dependent decrease in calculated CMRglu for the whole brain is another manifestation of the same phenomenon responsible for the discrepancy between measured and theoretically calculated [^{14}C]DG-6-P concentrations that is greatest at early times and then gradually diminishes and disappears by 45 min. This phenomenon was observed by Nelson et al. (1986*a*) and is also apparent in the results of Hawkins and Miller (1978) after their predicted time course of [^{14}C]DG-6-P accumulation is corrected for an appropriate lumped constant (Fig. 2). Measured [^{14}C]DG-6-P concentrations exceed theoretical concentrations at early times, but the difference diminishes with time and becomes negligible by 30–45 min. Hawkins and Miller interpret this phenomenon as evidence of G-6-Pase activity, but there is an obvious alternative explanation. This phenomenon is the consequence of the forced imposition of a single-compartment model on a multi-compartment tissue, such as freeze-blown brain. Complete agreement between measured and theoretical accumulations of [^{14}C]DG-6-P over the entire time course can be expected only if the tissue is completely homogeneous because the theoretical concentrations are calculated with the assumption of a homogeneous compartment with a single unique set of rate constants. The first explicitly stated assumption of the DG model is that it is "applicable only to a localized region of tissue that is homogeneous with respect to the following: rate of blood flow; rates of transport of [^{14}C]DG and glucose between plasma and tissue; concentrations of [^{14}C]DG, glucose, [^{14}C]DG-6-P, and G-6-P; and rate of glucose

utilization" (Sokoloff et al., 1977). A freeze-blown brain is a heterogeneous mixture of many compartments, each with its own unique rates of blood flow, blood–brain barrier transport of hexoses, glucose metabolism, and DG phosphorylation. These functions all tend to be positively correlated. The influences of these factors are reflected in the rate constants of the DG operational equation (Fig. 2B) (Sokoloff et al., 1977). Values for these rate constants vary widely among the macroscopic structures of the brain and among the microscopic components of each structure. There is no single set of rate constants that applies to the whole brain or to any heterogeneous part of it for the entire time course. The theoretical time course of [^{14}C]DG-6-P accumulation is calculated by assuming a single homogeneous compartment and a single set of rate constants operating throughout the experiment. The true mean rate of glucose utilization in the whole brain is the mean of the rates in all components, each weighted for its relative mass. The mean rate of [^{14}C]DG-6-P accumulation in whole brain is, however, weighted not only by the relative mass of each component but also by the history of the concentrations of the precursor in each of the compartments. Tissues with higher metabolic rates generally have higher rates of blood flow, transport, and [^{14}C]DG uptake and are, therefore, disproportionately more heavily represented in the mean rate of [^{14}C]DG-6-P accumulation at early times because of a more rapid uptake of precursor. With increasing time after a pulse, the slower compartments become progressively more represented, until eventually all the compartments become weighted in proportion to their relative masses within the mixed tissue (Fig. 3) (See Appendix 1 for mathematical proof). This progressive change in weighting explains why measured and theoretical time courses of [^{14}C]DG-6-P are different at early times but converge at later times after a pulse. The measured [^{14}C]DG-6-P accumulation at early times is excessively weighted by fast compartments and overestimates the mass-weighted mean rate for the mixed tissue. At later times, theoretical and measured values are similar because by then they both represent better the mean of all the compartments properly weighted for their relative masses.

Other similar observations cited by Hawkins and Miller as evidence of effects of G-6-Pase activity on [^{14}C]DG-6-P accumulation are also explained by the effects of heterogeneity. For example, Pelligrino et al. (1987) assayed [^{14}C]DG-6-P and [^{14}C]DG concentrations in serial samples of parietal cortex aspirated from goats at various times after a pulse of [^{14}C]DG. From the accumulation of [^{14}C]DG-6-P and the mean [^{14}C]DG concentration in the samples soon after the pulse, they calculated an apparent k_3^*, the rate constant for DG phosphorylation. When they used this rate constant to predict the subsequent time course of [^{14}C]DG-6-P accumulation, they found that the computed time course exceeded the measured accumulation. They attributed the discrepancy to G-6-Pase activity. The parietal cortex is, however, quite heterogeneous, not only at the microscopic level but also at the macroscopic level; this heterogeneity is obvious from stained histological sections or [^{14}C]DG autoradiographs. The apparent k_3^* they determined at early times is for a mixture of compartments and, unlike a k_3^* for a single homogeneous compartment, varies with time. At early times it is high because it is heavily weighted by the fast compartments, but it progressively decreases with time as the slower compartments become better represented (see Appendix 1). The use of a k_3^* determined in a mixed tissue at early times leads to overestimates of [^{14}C]DG-6-P concentrations at later times; conversely, the use of a k_3^* determined at late times leads to underestimates of [^{14}C]DG-6-P concentrations at early times. A simple analogy that illustrates the fallacy of their analysis is the determination of a half-life from an early segment of a multi-exponential curve and then using it to try to predict the subsequent time course of the curve. The effects of heterogeneity also contributed to

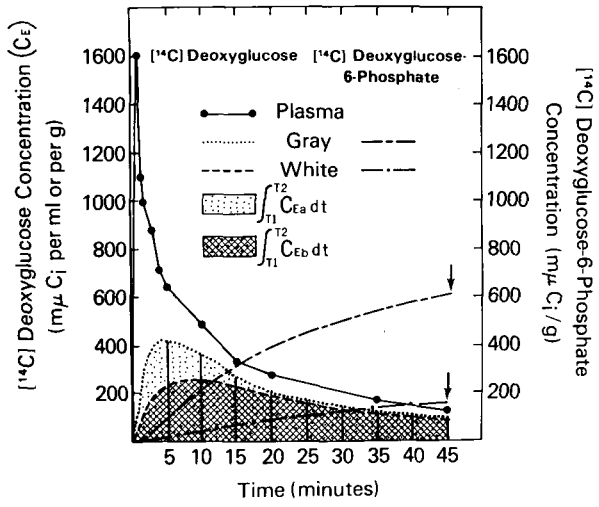

FIG. 3. A typical measured time course of arterial plasma DG concentration and theoretical time courses of DG and DG-6-P concentrations in gray matter and white matter expected in normal conscious rats. Differences in gray and white matter arise from differences in rate constants in the two types of tissues. The rate constants used in the computations are arithmetic means of those previously determined in gray structures ($K_{1a}^* = 0.189$; $k_{2a}^* = 0.245$; $k_{3a}^* = 0.052$) and white structures ($K_{1b}^* = 0.079$; $k_{2b}^* = 0.133$; $k_{3b}^* = 0.02$) (Sokoloff et al., 1977). Higher values for rate constants in gray matter than in white matter reflect higher rates of blood flow, blood–brain barrier transport, and DG metabolism than in white matter. Inasmuch as these values are *already* means of distributions of rate constants, they underestimate the wide diversity of rate constants within various structures in the brain; these extend over at least a three- to fivefold range making effects of heterogeneity even greater. C_{Ea} and C_{Eb} refer to DG concentrations in gray and white matter, respectively, and are calculated according to the second term in the numerator of the operational equation of the method (Fig. 2B) as described by Sokoloff et al. (1977). Modified from Fig. 5A of the report of Sokoloff et al. (1977).

the results of Huang and Veech (1985), who fitted a k_3^* and k_4^* to measured concentrations of [^{14}C]DG-6-P and [^{14}C]DG in freeze-blown whole rat brains at various times up to 45 min after a pulse of [^{14}C]DG and found an apparent $k_4^* > 5\%$/min; perhaps even more contributory to this extraordinarily high value was their incomplete recovery of [^{14}C]DG-6-P from brain because of its inadequate elution from Dowex-1-formate columns with 4 *M* formic acid (Dienel et al., 1986).

Although DG-6-P loss due to G-6-Pase will lead to a finite k_4^* in the fitting routines that are usually used to estimate the rate constants from measured time courses of DG and DG-6-P concentrations in tissue and plasma, the finding of a finite k_4^* does not prove that G-6-Pase activity is responsible. All the models used to fit the rate constants assume a constant k_3^* throughout the time course. If the apparent k_3^* decreases with time, as it does in a mixture of fast and slow compartments, the decrease is manifested as a finite k_4^*. Given the limited spatial resolution of PET scanners (8–20 mm), it is almost certain that heterogeneity contributes to the magnitude of the k_4^* values that have been found and to the overestimation of the influence of G-6-Pase activity.

The influences of blood flow, blood–brain barrier transport, turnover of the precursor pool, and heterogeneity on estimates of metabolic rates derived from measurements of precursor in plasma and tracer uptake into the tissue are all taken into account by the rate constants in the exponential terms in the operational equation (Fig. 2B). These effects are minimized when the exponential terms can be reduced to levels that minimally affect the solution of the equation; this is, in fact, why DG was selected over glucose for measuring LCMRglu. After a pulse of tracer, the plasma level progressively decreases, and the values of the exponential terms in the equation also decrease; at the same time the terms from which the exponential terms are subtracted grow larger. Eventually the exponential terms become so small that they have relatively little influence on the final result. With DG, it is possible to prolong the experimental period to 45 min without a significant loss of product, thus allowing the exponential terms to decline to low enough levels to minimize the influence of heterogeneity and all other processes reflected in the rate constants. With glucose it is necessary to so restrict the experimental period (because of much earlier loss of product) that the exponential terms remain substantial. Large errors can then result from inaccuracies in the rate constants, which vary with the structure, animal, experimental condition, and time (because of heterogeneity) and cannot be determined simultaneously with the measurement of LCMRglu. The 45-min period of the DG procedure is long enough to minimize the influence of the rate constants and yet short enough to avoid serious consequences of G-6-Pase activity (Sokoloff, 1982).

REVERSAL OF CEREBRAL ARTERIOVENOUS DIFFERENCE FOR DG

Hawkins and Miller repeat the argument of Sacks et al. (1983) that the reversal of the cerebral arteriovenous difference for [^{14}C]DG, but not for [^{14}C]glucose, 5–10 min after an intravenous infusion indicates that earlier formed [^{14}C]DG-6-P is being hydrolyzed and the label is being released as [^{14}C]DG. Nelson et al. (1986*a*) showed that the reversal depends on the equilibrium distribution ratio for the precursor between tissue and plasma, the ratio of k_2^* (the rate constant for transport from brain to plasma) to k_3^* (the rate constant for phosphorylation by hexokinase), and the arterial input function. There is no reason to invoke G-6-Pase activity to explain the reversal. The reversal occurs when the declining plasma level falls so much faster than the tissue level that the tissue/plasma concentration ratio exceeds the equilibrium distribution ratio. At that point net outward transport ensues. This situation is more easily achieved with DG than with glucose because its k_2^* is about five times greater than its k_3^*, and its equilibrium distribution ratio is more than twice that of glucose; the two rate constants are nearly equal for glucose. Nelson et al. (1986*a*) showed the effects of these differences with a simple simulation analysis. Hawkins and Miller question the accuracy of the rate constants used in the computations, but the rate constants need not be accurate for this phenomenon to be observed; all that is required is that the k_3 for glucose phosphorylation be sufficiently greater than the k_3^* for DG phosphorylation (See Fig. 3B of Nelson et al., 1986*a*), an obvious consequence of three to fourfold higher affinity of brain hexokinase for glucose than for DG (Grossbard and Schimke, 1966). Hawkins and Miller further state: "Their calculations seem to conflict with data in the article by Sokoloff et al. (1977, Fig. 5) which predict the total radioactivity in brain—[^{14}C]DG plus [^{14}C]DG-6-P—to rise continuously. Since net accumulation can occur only when the arteriovenous difference is positive, the occurrence of substantial negative differences beginning between 5 and 10 min stands in contradiction." It would indeed be contradictory if the two results were obtained in the same animal at the same time. Whether or when the arteriovenous difference is reversed depends also on the shape of the arterial input function; the longer the period of injection of the tracer and/or the more rapid the decline in arterial concentration after the injection ends, the more likely and sooner reversal will appear. With a sharp, early peak followed by a relatively slow fall in arterial concentration, reversal may never occur. In Fig. 5 of Sokoloff et al. (1977) the arterial input was for an animal in a typical DG experiment, whereas in Fig. 3 of Nelson et al. (1986*a*) the rising phase of the arterial curve for both DG and glucose was more prolonged to simulate more closely the arterial inputs of Sacks et al. (1983) with which reversal with [^{14}C]DG, but not [^{14}C]glucose, was seen.

REGIONAL G-6-PASE ACTIVITY IN BRAIN

Hawkins and Miller cite an abstract by Hawkins et al. (1986) that claimed to show a gradient of increasing G-6-Pase activity from telencephalon to brainstem; regions with higher activities were those that had lower values for LCMRglu determined with [^{14}C]DG than with [6-^{14}C]glucose. The G-6-Pase activities in the brain regions were not assayed, but were estimated on the basis of the loss of ^{14}C from regions of brain 45–240 min after the brain had been labeled in vivo with [^{14}C]DG. These estimates may be as unreliable as those we made 17–24 h after a pulse of [^{14}C]DG (Sokoloff et al., 1977) because of the incorporation of label into slowly turning over pools, such as glycogen (Nelson et al., 1984). Indeed, Anchors and Karnovsky (1975) found no such regional distributions of G-6-Pase activity directly assayed in brain. Also, in the series in Table 1 and Fig. 1 and in two recent series (Mori et al., 1986), there were no significant differences between LCMRglu determined with the [^{14}C]DG method at 30 and 45 min in structures claimed by Hawkins et al. (1986) to be most affected by G-6-Pase activity; significant G-6-Pase activity would have led to lower values for LCMRglu at 45 than at 30 min. The differences between values for LCMRglu found by Hawkins et al. (1986) with the [^{14}C]DG and [^{14}C]glucose methods in some brain regions are probably more indicative of problems with the [^{14}C]glucose method than with the [^{14}C]DG method. The past decade has seen three, possibly four, generations of [^{14}C]glucose methods (Hawkins et al., 1974, 1979, 1985; Lu et al., 1983); each has been presented as definitive, yet each has provided values for LCMRglu progressively approaching those obtained with the [^{14}C]DG method. Moreover, none of these methods has yet adequately addressed: (1) integrated precursor-specific activities in local regions during the experimental period; (2) influences of blood flow and transport on these essential variables; (3) local residual pools of free [^{14}C]glucose at the time of killing; (4) local losses of labeled products of [^{14}C]glucose metabolism; (5) effects of uptake into some brain regions of recirculating, systemically produced labeled metabolites of [^{14}C]glucose; or (6) effects of heterogeneity in structures examined in the short experimental period required with [^{14}C]glucose.

GLUCOSE-CHASE EXPERIMENTS

Hawkins and Miller described a glucose-chase experiment that demonstrated DG-6-P loss between 20 and 45 min. They administered identical pulses of [^{14}C]DG to rats and killed half at 20 min by freeze-blowing the brain. The remaining rats were given large intravenous doses of unlabeled glucose (sufficient to inhibit further uptake and phosphorylation of [^{14}C]DG by brain) at 20 min and killed similarly at 45 min. Assays of brain [^{14}C]DG-6-P concentrations showed that animals with the glucose-chase killed at 45 min had 19% less brain DG-6-P than those killed at 20 min, suggesting an appreciable loss of DG-6-P within 20–45 min after the pulse. This study is documented by an abstract (Hawkins et al., 1986) that makes no mention of such experiments. Because of the critical nature of these results and the absence of published information on which to judge them, we performed similar experiments. Twenty-seven rats were administered identical doses of [^{14}C]DG (125 μCi/kg; 55 μCi/μmol). Nine were killed at 20 min and six at 45 min. In eight animals the arterial plasma glucose concentration was clamped at 20 min at a level of 29–34 mM (three to four times the level existing during the first 20 min) (DeFronzo et al., 1979) and maintained constant between 20 and 45 min. At

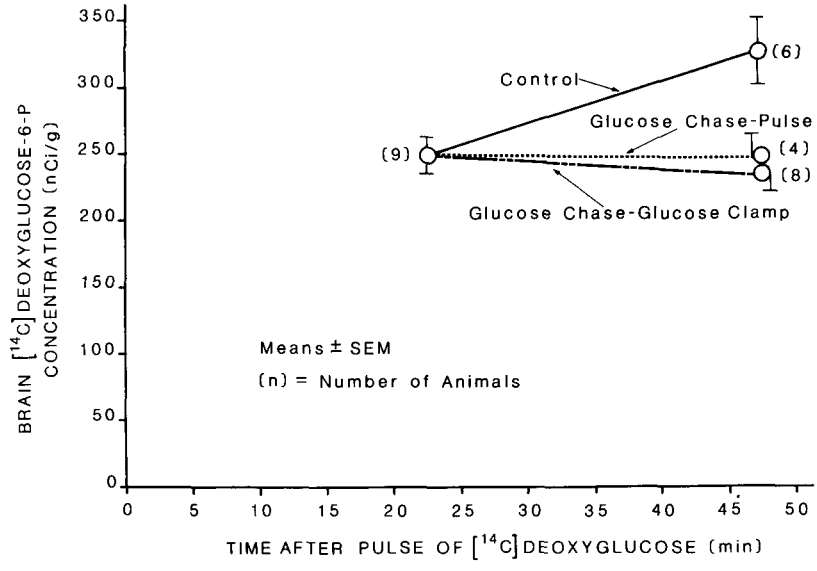

FIG. 4. Accumulation of DG-6-P in brains of control rats, in rats given an intravenous pulse of 8 mmol of glucose at 20 min, and in rats with plasma glucose concentration clamped at 29–34 mM between 20 min and kill time.

20 min, four animals were given intravenous pulses of glucose (approximately 8 mmol/kg) sufficient to raise the plasma levels transiently to 100–170 m*M*. Animals with a glucose-chase were killed at 45 min. All animals were killed by freeze-blowing the brains, which were assayed for [^{14}C]DG-6-P content. In animals without a glucose-chase, [^{14}C]DG-6-P content rose between 20 and 45 min (Fig. 4). Both types of glucose-chase blocked any further accumulation of [^{14}C]DG-6-P in brain between 20 and 45 min (Fig. 4). In neither group of animals with glucose-chases and killed at 45 min were the brain [^{14}C]DG-6-P contents significantly different from that of the animals killed at 20 min (Fig. 4). We cannot, therefore, confirm that a glucose-chase uncovers significant loss of DG-6-P from brain during the prescribed 45-min experimental period of the DG method.

In short, Hawkins and Miller present no new evidence to refute repeated experimental demonstrations that G-6-Pase activity has no significant effect on the results obtained with the DG method when used as prescribed. Indeed, a critical and objective review of all the literature emanating from more than 10 years of experience with the DG method strongly confirms its validity.

APPENDIX I: EFFECTS OF HETEROGENEITY ON KINETICS OF DG-6-P ACCUMULATION

Assumptions

1. Tissue samples assayed for DG and DG-6-P contents are a heterogeneous mixture of at least two homogeneous compartments, *a* and *b* (in reality, there are many).
2. There is no loss of DG-6-P from tissue due to G-6-Pase activity.

Definitions of symbols

a: Fast tissue compartment with high rates of blood flow, DG transport between plasma and tissue, and DG phosphorylation (i.e., high values for K_1^*, k_2^*, and k_3^*).

b: Slow tissue compartment with low rates of blood flow, DG transport, and phosphorylation (i.e., low values for K_1^*, k_2^*, and k_3^*).

Q_{ma} = quantity of DG-6-P in compartment *a* in tissue sample.

Q_{mb} = quantity of DG-6-P in compartment *b* in tissue sample.

Q_{mT} = measured total quantity of DG-6-P in both compartments, *a* and *b*, contained in total tissue sample.

Q_{Ea} = quantity of free DG in compartment *a* in tissue sample.

Q_{Eb} = quantity of free DG in compartment *b* in tissue sample.

Q_{ET} = measured total quantity of free DG in both compartments *a* and *b*, contained in total tissue sample.

C_{ma} = concentration of DG-6-P in compartment *a*.

C_{mb} = concentration of DG-6-P in compartment *b*.

$\bar{C}_m$ = measured mass-weighted mean concentration of DG-6-P in total tissue sample.

C_{Ea} = concentration of free DG in compartment *a*.

C_{Eb} = concentration of free DG in compartment *b*.

$\bar{C}_E$ = measured mass-weighted mean concentration of free DG in total tissue sample.

W_T = total mass of tissue sample.

W_a = mass of compartment *a* in tissue sample.

W_b = mass of compartment *b* in tissue sample. Therefore, $W_T = W_a + W_b$ and $W_a/W_T + W_b/W_T = 1$

k_{3a} = rate constant for phosphorylation of DG in compartment *a*.

k_{3b} = rate constant for phosphorylation of DG in compartment *b*.

"$\bar{k}_3$" = apparent mean rate constant for phosphorylation of DG in heterogeneous mixture of compartments *a* and *b* in total tissue sample.

$\bar{k}_3$ = true mass-weighted mean rate constant for phosphorylation of DG in heterogeneous mixture of compartments in tissue sample.

$T1$ = any time $\geqq 0$.

$T2$ = any time $> T1$.

Mathematical analysis

By Law of Conservation of Matter,

$$Q_{mT} = Q_{ma} + Q_{mb} \quad \text{and} \quad Q_{ET} = Q_{Ea} + Q_{Eb}$$

But Quantity (Q) = Concentration per unit mass (C) × Mass (W). Therefore,

$$W_T\bar{C}_m = W_aC_{ma} + W_bC_{mb} \tag{1}$$

$$W_T\bar{C}_E = W_aC_{Ea} + W_bC_{Eb} \tag{2}$$

and

$$\bar{C}_m = (W_a/W_T)C_{ma} + (W_b/W_T)C_{mb} \tag{3}$$

$$\bar{C}_E = (W_a/W_T)C_{Ea} + (W_b/W_T)C_{Eb} \tag{4}$$

The rates of change of concentration of DG-6-P in compartments *a* and *b* at any instant of time are represented by

$$dC_{ma}/dt = k_{3a}C_{Ea} \quad \text{and} \quad dC_{mb}/dt = k_{3b}C_{Eb}$$

and the changes in concentration of DG-6-P in compartments *a* and *b* that occur during any interval of time, $T1$ to $T2$, are obtained by integration of these equations between $T1$ and $T2$. Thus,

$$\Delta C_{ma}|_{T1}^{T2} = k_{3a}\int_{T1}^{T2} C_{Ea}dt$$

$$\text{and} \quad \Delta C_{mb}|_{T1}^{T2} = k_{3b}\int_{T1}^{T2} C_{Eb}dt \tag{5}$$

From Eq. 3 the mean concentration of DG-6-P in the total mixed tissue equals the mass-weighted concentrations of DG-6-P in all compartments. The change in the mean concentration of DG-6-P in the mixed tissue must, therefore, also be the mass-weighted mean of the changes in the individual compartments. Therefore, combining Eqs. 3 and 5 yields

$$\Delta\bar{C}_m|_{T1}^{T2} = (W_a/W_T)k_{3a}\int_{T1}^{T2} C_{Ea}dt + (W_b/W_T)k_{3b}\int_{T1}^{T2} C_{Eb}dt \tag{6}$$

The mean concentration in the mixed tissue at any time, $T2$, after zero time is obtained by integrating from zero time to $T2$.

$$\bar{C}_m(T2) = (W_a/W_T)k_{3a}\int_{T0}^{T2} C_{Ea}dt + (W_b/W_T)k_{3b}\int_{T0}^{T2} C_{Eb}dt \quad (7)$$

It is clear that both the concentration and rate of change of concentration of DG-6-P in a mixed tissue are determined by the individual compartment rate constants for DG phosphorylation, each weighted not only by the relative mass of its compartment but also by the integrated concentration of DG that existed in that compartment over the given interval of time. Figure 3 shows that both the actual and integrated concentrations of DG are greater in fast compartments than in slow compartments at early times, and that these values in the fast and slow compartments only gradually approach equality with time. The overall rate of DG-6-P accumulation in a mixture of compartments therefore reflects at first mainly the rates of accumulation in the fastest compartments. With increasing time, when the integrated DG concentrations in the various compartments approach equality (Fig. 3), events in the slower compartments become equally weighted in the mean. It is therefore only at late times that the rate of DG-6-P accumulation in a mixed tissue represents the true mean of the rates in all the compartments properly weighted for their relative masses in the mixed tissue. For example, when

$$\int_{T1}^{T2} C_{Ea}dt = \int_{T1}^{T2} C_{Eb}dt = \int_{T1}^{T2} \bar{C}_E dt,$$

which occurs relatively late after a pulse of DG (e.g., 20–30 min) (see Fig. 3), the integrated DG concentration in the compartments can be factored out, and Eq. 6 becomes

$$\Delta\bar{C}_m|_{T1}^{T2} = [(W_a/W_T)k_{3a} + (W_b/W_T)k_{3b}]\int_{T1}^{T2} \bar{C}_E dt \quad (8)$$

It is only then that a true mean $\bar{k}_3$ for the total mixed tissue that is constant with time and appropriately weighted only for the relative masses of the compartments is obtained. Thus, at late times,

$$\text{True } \bar{k}_3 = [(W_a/W_T)k_{3a} + (W_b/W_T)k_{3b}] \quad (9)$$

and

$$\Delta\bar{C}_m|_{T1}^{T2} = \bar{k}_3\int_{T1}^{T2} \bar{C}_E dt \quad (10)$$

Before that time there is no true constant mean $\bar{k}_3$ for the mixed tissue. There is only an apparent "$\bar{k}_3$" that decreases with time as the initially disproportionately greater weighting of the faster compartments declines, slower compartments become more properly represented, and eventually the various rates in all compartments contribute to the overall mean of the mixture only in proportion to their relative masses in the tissue.

Further proof that the apparent "$\bar{k}_3$" varies with time because of time-dependent changes in weighting of the various compartments due to differences in DG concentrations is as follows: k_3 is defined as the rate constant for DG phosphorylation in a homogeneous compartment where

$dC_m/dt = k_3C_E$. Integrating between $T1$ and $T2$,

$$\Delta C_m|_{T1}^{T2} = k_3\int_{T1}^{T2} C_E dt \quad \text{and} \quad k_3 = \Delta C_m|_{T1}^{T2} \Big/ \int_{T1}^{T2} C_E dt$$

But for a mixture of compartments, a and b, weighted means of $\bar{C}_E$, as defined by Eq. 4, and of $\bar{C}_m$, as defined by Eq. 6, must be substituted. Thus,

$$\text{Apparent "}\bar{k}_3\text{"} = \frac{(W_a/W_T)k_{3a}\int_{T1}^{T2} C_{Ea}dt + (W_b/W_T)k_{3b}\int_{T1}^{T2} C_{Eb}dt}{(W_a/W_T)\int_{T1}^{T2} C_{Ea}dt + (W_b/W_T)\int_{T1}^{T2} C_{Eb}dt} \quad (11)$$

It is obvious then that the apparent "$\bar{k}_3$" for a mixture of compartments varies with time because of the time-dependent changes in the proportions of the weighting factors, $\int_{T1}^{T2} C_{Ea}dt$ and $\int_{T1}^{T2} C_{Eb}dt$. At early times the compartments with the highest rates of uptake and phosphorylation of DG predominate; at later times the compartments are more equally represented (Fig. 3). It is only at late times, when $\int_{T1}^{T2} C_{Ea}dt$ and $\int_{T1}^{T2} C_{Eb}dt$ become equal (Fig. 3) and can be factored out and cancelled out, that

$$\text{Apparent "}\bar{k}_3\text{"} = \text{true } \bar{k}_3 = (W_a/W_T)k_{3a} + (W_b/W_T)k_{3b} \quad (12)$$

This analysis shows that it is impossible to fit a theoretical time course for DG-6-P accumulation in a mixed tissue, such as whole brain or even a part of brain, to measured concentrations over the entire time course with a model based on a single homogeneous compartment and a single, constant set of rate constants. Measured concentrations of DG-6-P are weighted means of all concentrations in all compartments, each weighted according to its relative mass and the history of the free DG concentration in the compartment preceding the time of measurement. At early times, fast compartments are disproportionately (i.e., with respect to their relative masses) more heavily weighted than slow compartments (Fig. 3). Predictions of the time course at late times made from measurements at early times leads to overestimates of the expected DG-6-P accumulation above measured values because with increasing time, slower compartments become more appropriately represented in the measured mean concentration. It is only at later times, when the integrated DG concentrations in all compartments approach equality (Fig. 3), that the measured accumulation of DG-6-P in a mixed tissue begins to reflect the true weighted mean metabolic rate in all compartments properly weighted in proportion to their relative masses in the tissue.

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